Indian Journal of Geo Marine Sciences
Vol. 49 (03), March 2020, pp. 471-476

Short Communication

Isolation of bioluminescent bacteria from marine organisms

Paritosh Parmar, Arpit Shukla, Meenu Saraf & Baldev Patel*

Department of Microbiology & Biotechnology, University School of Sciences, Gujarat University, Ahmedabad-380 009, India

*[E-mail: patelbaldev56@yahoo.in]

Received 31 July 2018; *05 October 2018*

Bioluminescence is an emission of cold light by enzyme driven reaction within certain living organisms. The most abundant and widely distributed light emitting organisms are luminescent bacteria. Such organisms are either found as free-living in the ocean or in symbiotic relationship with the marine host. To employ bioluminescence in environmental monitoring, isolation of bioluminescent bacteria from the two different marine samples (sea water sample and various organs of squid and fish) were collected from different sites of Veraval seashore and fish markets located nearby seashore respectively. The marine organisms used in the study were 20-25 days old. Cultivation media that were used for isolation were sea water agar (SWA), luminous agar (LA) and nutrient agar (NA); out of which SWA proved to be the most suitable medium for their growth and luminescence. No bioluminescent bacterium was found in water samples and total five bioluminescent bacteria were isolated from five different organs of fish and squid each. Out of these five isolates, two were selected based on their maximum light intensity. These two isolates, PBS1 and PBF1, were further characterized biochemically. PBS1 was able to utilize glucose, galactose, maltose and were tested positive for catalase and oxidase tests. Similar results were obtained in case of PBF1 except it was tested positive for urease urea but was unable to utilize glucose. Both isolates thrived at neutral pH and showed maximum bioluminescence. Effect of NaCl concentration on luminescence revealed that the two isolates were not able to grow in media devoid of NaCl and the luminescence was found to be maximum at 3 % (w/v) NaCl supplementation.

[Keywords: Bioluminescence, Bioluminescent bacteria, Fish, *Photobacterium*, Squid, *Vibrio*]

Introduction

Bioluminescence is an emission of low wavelength light inclining towards the blue-green part of the electromagnetic spectrum by an enzyme driven reaction within certain living organisms[1]. It is distributed in diverse group of organisms ranging from aquatic organisms such as dinoflagellates, fishes, squids, including free living bacteria to terrestrial invertebrates like fireflies and mushrooms[1-5]. Among these organisms, the most abundant and widely distributed light emitting organisms are luminescent bacteria. Such bioluminescent bacteria are either found in planktonic form in the ocean[6-12] or in symbiotic relationship with the marine host for instance certain fishes and squids[2,8,13-17].

The reaction which is responsible for emission of light involves the enzyme luciferase that oxidizes long chain aliphatic aldehyde (RCHO), reduced flavin mononucleotide ($FMNH_2$), and oxygen (O_2) to a long chain aliphatic acid (RCOOH), oxidized flavin mononucleotide (FMN), and water (H_2O). In this process, the excess energy is liberated and emitted as luminescent blue-green light of 490 nm (Fig. 1)[4]. All luminescent bacteria are distributed among three genera *Vibrio*, *Photobacterium*, *Xenorhabdus*[4,18]. However, recently one more genera *Kurthia* has also been reported to be luminescent[19]. These bacteria possess a gene sequence, known as *lux* operon. In *Vibrio fischeri*, *lux* operon consists of *luxCDABE* genes, of which, *luxCDE* encodes proteins for the synthesis of aldehyde, *luxA* and *luxB* encodes subunits of bacterial luciferase[20]. Bioluminescent bacteria are very sensitive to various toxic or hazardous compounds. Therefore, attempts have been made to apply this property to various fields of environmental monitoring using either, natural isolate[21] or by generating recombinant bioluminescent bacteria[22-24].

Biological materials such as; enzyme, antibody and antigen, are known to be highly specific in nature. Thus, it has wide applications in the field of biological environmental surveillance. Present study was carried out to isolate potential bioluminescent bacteria from marine organisms, in order to characterize, optimize and potentially apply them in environmental monitoring.

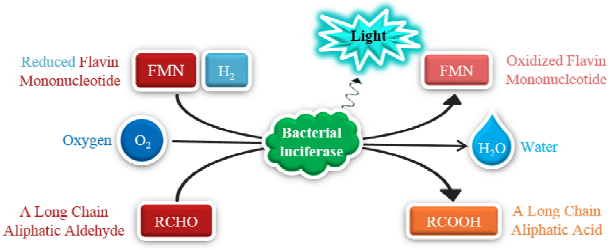

Fig. 1 — Emission of bioluminescence by bacterial luciferase[4]

Materials and Methods

Sample Collection

Sea water samples were collected from two different locations of coastal regions of Gujarat, which were; Veraval seashore (DMS: 20° 53' 54.1" N, 70° 22' 37.6" E) and Somnath seashore (DMS: 20° 53' 01.9" N, 70° 24' 18.4" E). These samples were collected in sterile collection bottles. Two marine organisms were short listed for the isolation of bioluminescent bacteria, one was Indian mackerel (*Rastrelliger kanagurta*) and another was a squid (*Loligo duvauceli*). These two samples were procured from the fish market, located nearby of Veraval seashore. Both kinds of sample were stored at 4 ± 2° C until further processing.

Isolation

Isolation was performed on three different media, nutrient agar (NA) (g/L: peptone 5.0, NaCl 5.0, HM peptone B 1.5, yeast extract 1.5, agar 15.0, pH 7.4 ± 0.2), sea water agar (SWA) (g/L: *Part A*: yeast extract 5.0, peptic digest of animal tissue 5.0, beef extract 3.0, agar 15.0; *Part B*: NaCl 24.0, KCl 0.7, $MgCl_2 \cdot 6H_2O$ 5.3, $MgSO_4 \cdot 7H_2O$ 7.0, $CaCl_2$ 0.1, pH 7.5 ± 0.2) and luminescent agar (LA) (g/L: NaCl 10.0, peptone 10.0 and yeast extract 5.0, agar powder 30.0, distilled water 1.0). Sea water samples were serially diluted and then inoculated by spread plate technique. To isolate luminescent bacteria, five organs were chosen from each marine organism (Table 1).

Both marine organisms were dissected ventrally, fish were dissected in a 'T' manner. Microflora from these organs was collected using sterile cotton swabs. These swabs were streaked on above mentioned media under aseptic condition. All the inoculated plates were incubated at 25 ± 2° C for 18-24 h. After incubation period plates were observed in complete dark condition at room temperature (25 ± 2° C) for identifying luminescent colonies. Such colonies were further sub-cultured for pure culture. Screening was done on the basis of intensity of luminescence of isolated bioluminescent bacteria for further study.

Table 1 — List of organs that were used for isolation

Sample	Organ
Squid (A)	Ink Sac
	Tentacles
	Eye portion
	Gills
	Skin
Fish (B)	Gut
	Gills
	Intestine
	Eye portion
	Scales

Characterization

For characterization, the experiments were carried out in sea water broth medium (g/L: yeast extract 5.0, peptic digest of animal tissue 5.0, beef extract 3.0, NaCl 24.0, KCl 0.7, $MgCl_2 \cdot 6H_2O$ 5.3, $MgSO_4 \cdot 7H_2O$ 7.0, $CaCl_2$ 0.1, pH 7.5 ± 0.2).

Activation of cultures

The log phase culture of each isolate, 50 mL SWB medium was inoculated with single colony respectively and incubated at 25 ± 2° C for 18-24 h on orbital shaker at 120 rpm.

Inoculation and incubation

For further study, the broth media were inoculated with 0.1 % (v/v) log phase culture of selected isolates (PBS1 and PBF1). In case of agar media loopful of the log phase culture was streaked on the surface of agar media. All the inoculated media were incubated on orbital shaker at 120 rpm, 25 ± 2° C for 24 h while media containing agar were incubated in static condition.

Assessment of luminescence

After incubation period, growth (if any) was observed in a dark room for luminescence. To record results, intensity of luminescence was noted in terms of visual assessment ('-': Negative, '+': Poor, '++': Good).

Microbial metabolism

For preliminary identification and understanding of metabolic activities of selected isolates, biochemical tests were carried out. Such biochemical tests can serve the basis for tentative classification and identification of the isolates. These tests were categorized as; [A] Based on carbon source: (i) fermentation of simple sugars such as glucose, galactose, sucrose, and maltose by using peptone water (HiMedia, India) with respective sugar discs (HiMedia, India). (ii) Tests based on glucose breakdown product such as methyl red and Voges-Prauskauer were performed to study the fate of glucose using glucose phosphate broth. (iii) The ability to utilize starch, lipid and citrate was tested using 1 % (w/v) starch agar, tributyrene agar and Simmon's citrate agar medium respectively. and

nutrient gelatin broth respectively. [B] Based on nitrogen source: (i) indole production was detected by 1 % tryptone broth, (ii) urea utilization was tested using urea broth, (iii) casein and gelatin hydrolysis ability was evaluated using milk agar medium and nutrient gelatin medium. (iv) deaminase enzyme production ability was tested using phenylalanine agar medium. Furthermore, [C] other tests such and catalase and oxidase were carried out determine the respiratory nature of the isolates using nutrient agar medium and oxidase disc (HiMedia, India).

Effect of environmental factors on the luminescence

Effect of pH

100 mL of SWB medium was prepared at 5 pH levels (3, 5, 7, 9, and 11). Acidic pH was adjusted using 0.1 N H_2SO_4 and alkaline pH was adjusted by 0.1 N NaOH solution with the help of Welltronix® AUTO pH SYSTEM PM300. The final volume of each medium was made up to 50 ml in 50 ml volumetric flask with distilled water, mixed well and transferred to 100 ml Erlenmeyer flasks.

Effect of NaCl Concentration (% w/v)

100 mL SWB medium was prepared by supplementing NaCl a t5 levels (0, 3, 6, 9, and 12) individually.

Results and Discussion

Isolation

Widely diverse colonies were observed on all three media, inoculated with water samples. When plates were observed in a dark room for luminescence, no such colony was obtained, even after 48 h of incubation. However, bioluminescent species *Vibrio* has been isolated from the water samples collected from different seashores of India. Bioluminescent *Vibrio* sp. were isolated from surface water collected from Diu beach (Gujarat), Nagapattinam seashore (Tamil Nadu), Dapoli beach (Maharashtra)[9,25,26]. On the other hand, bioluminescent colonies were obtained on agar plates which were inoculated with microflora of internal organs of squid and fish (Table 2). On the basis of the intensity of luminescence two isolates, PBS1 and PBF1 (Figs. 2 and 3) were screened in for further study. In accordance with the results so obtained in the present investigation, bioluminescent bacteria were isolated from fishes such as yellow striped scad, gut of Indian mackerel and gills of Threadfin bream[13,16]. Most of the isolation studies have revealed that bioluminescent *Vibrio* spp., usually found as free living in marine ecosystems, in close symbiotic relationship with Hawaiian bobtail squid and with other species of squids as well[27-29]. There are reports that demonstrated the isolation of

Table 2 — Isolation of Bioluminescent bacteria

Sample		Bioluminescent bacteria		Isolate
		Presence	No. of Isolate	
SEA WATER				
Veraval Seashore		-	-	-
Somnath Seashore		-	-	-
MARINE ORGANISMS				
Squid	Ink sac	+	01	PBS1
	Eye	-	-	-
	Tentacles	-	-	-
	Gill	+	01	PBS2
	Skin	+	01	PBS3
	Gill	-	-	-
	Gut	+	01	PBF1
Fish	Eye	-	-	-
	Scales	-	-	-
	Intestine	+	01	PBF2

'-': Negative, '+': Positive

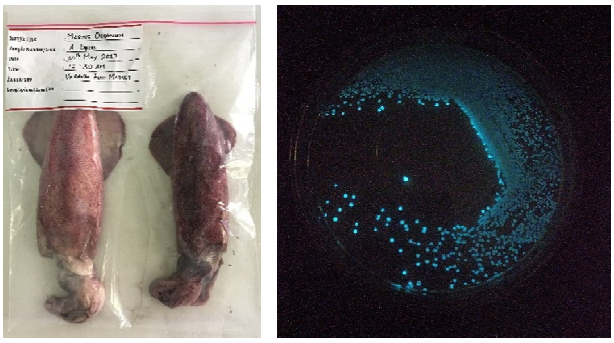

Fig. 2 — Squid samples and PBS1

Fig. 3 — Fish samples and PBF1

Photobacterium spp. from marine fishes such as salmon and ponyfish[30].

Microbial metabolism on the basis of biochemical tests

Selected bioluminescent bacteria were identified on the basis of their biochemical ability to utilize or hydrolyze particular substrates (Table 3). Catalase and oxidase tests were used to determine aerobic or anaerobic nature of the bacteria. It is well established that catalase is produced by aerobic or facultative anaerobic bacteria but not obligate anaerobes. Similarly, oxidase test detects the presence of cytochrome oxidase which is the last link in respiratory chain where electrons are passed on to the terminal electron acceptor, oxygen. Both isolates, PBS1 and PBF1, were found to be positive for catalase and oxidase tests indicating highly aerobic nature of these isolates. Such nature of bacteria can be explained by the biochemical reaction of bioluminescence where molecular oxygen is directly involved in the reaction catalyzed by luciferase enzyme. In addition, oxidase test is also used to differentiate gram negative pathogenic species such as *Neisseria gonorrhea*, *Pseudomonas aeruginosa* and *Vibrio* spp. from the species of family *Enterobacteriaceae* which are oxidase negative[31]. Using Bergey's Manual® of systematic bacteriology, isolate PBS1 and PBF1 may be identified as *Photobacterium leiognathi* and *V. Fischeri* respectively[32]. However, this classification is still partial, for complete classification further tests will be required.

Table 3 — Results of biochemical tests

	Isolates	
Test	PBS1	PBF1
Glucose	+	-
Galactose	+	+
Sucrose	-	-
Maltose	+	+
Starch utilization	-	-
Casein hydrolysis	-	-
Gelatin hydrolysis	-	-
Lipid utilization	-	-
Citrate utilization	-	-
Phenylalanine deamination	-	-
Indole production	-	-
Methyl red	-	-
Voges-Proskauer	-	-
Urea hydrolysis	-	+
Catalase	+	+
Oxidase	+	+

'-': Negative, '+': Positive

Effect of pH on luminescence

pH 7 was found to be optimum for both the good luminescence of the isolates. However, these two isolates were unable to grow at pH 3 and 11. Interestingly, isolate PBS1 was able to emit low intensity of luminescence at pH 9 whereas; isolate PBF1 was failed to grow beyond pH 7 (Table 4).

Effect of salt (NaCl) concentration on luminescence

Bioluminescent bacteria require significantly higher salt concentration owing to the fact their source of isolation being marine ecosystem. Such bacteria fail to grow in the absence NaCl probably failing to grow in low osmotic stress. Present study is in agreement with the aforementioned notion and the isolates PBS1 and PBF1, failed to grow in absence of NaCl. At 3 % NaCl concentration luminescence of both the isolates was observed to be maximum but at 6 % there was not any change in luminescence detected in isolate PBF1. Such observation suggest that NaCl plays an important role in growth stimulation. Further increase in NaCl concentration has a negative effect on luminescence (Table 5). Similar results obtained in case of bioluminescent *Photobacterium* sp. strain LuB-1 displayed maximum luminescence at 3 % (w/v) NaCl concentration indicating the dependence of growth and luminescence of the organism on amount of

Table 4 — Effect of pH on luminescence

	Isolates	
pH	PBS1	PBF1
3	-	-
5	+	+
7	++	++
9	+	-
11	-	-

'-': Negative, '+': Poor, '++': Good

Table 5 — Effect of salt (NaCl) concentration on luminescence

	Isolates	
NaCl Conc. (%)	PBS1	PBF1
0	-	-
3	++	++
6	+	++
9	-	-
12	-	-

'-': Negative, '+': Poor, '++': Good

NaCl present in the sample[33]. However, it was reported that NaCl may act as a stress factor for luminescence suggesting that an increase in NaCl concentration eventually diminishes the luminescence[28].

Conclusion

Bioluminescent bacteria were successfully isolated from squid and fish. Planktonic bioluminescent bacteria were not obtained from sea waters samples. Preliminary studies suggest that the selected two bacteria, PBS1 and PBF1, may be identified as *Photobacterium* sp. PBS1 (*P. leiognathi*) and *Vibrio* sp. PBF1 (*V. fischeri*), respectively. Both strains showed maximum light intensity at pH 7 and 3 % NaCl concentration. However, light intensity of PBF1 at 6 % NaCl concentration was not affected. This bioluminescent property of isolates can be further utilized to develop bio-monitoring tools or biosensors.

References

1 Hastings, J. W., Bioluminescence in bacteria and dinoflagellates, In: *Light emission by plants and bacteria,* Edited by Govindjee, J. Amesz and D. Fork. (Academic Press, inc.: London) 1986, 363-398.

2 Hastings, J. W., Potrikusv, C. J., Gupta, S. C., Kurfürst, M., & Makemson, J. C., Biochemistry and physiology of bioluminescent bacteria, In: *Advances in Microbial Physiology* (Academic Press, Inc.: London) 1985, 235-291.

3 Harvey, E. N., Luminescent organisms, *Am. Sci.*, 40 (1952) 468-481.

4 Nealson, K., & Hastings, J. W., Bacterial bioluminescence: its control and ecological significance, *Microbiol. Reviews*, 43 (1979) 496-518.

5 Weitz, W. H. J., Naturally bioluminescent fungi, *Mycologist*, 18 (1) (2004), 4-5.

6 Arulmoorthy, M. P., Karunakaran, K., Vignesh, R., Vasudevan, S., & Srinivasan, M., Identification and antimicrobial potential of bioluminescent bacteria isolated from the mangrove ecosystem of the Roach Park, Tuticorin, South east coast of India, *BMR Microbiology*, 1 (2014) 1-15.

7 Karaś, K., Sałkowska, A., Walczak-Drzewiecka, A., Ryba, K., Dastych, J., Bachorz, R. A., & Ratajewski, M., The cardenolides strophanthidin, digoxigenin and dihydroouabain act as activators of the human RORγ/RORγT receptors, *Toxicol Lett.*, 295 (2018) 314-324.

8 Jabalameli, L., Razavi, M. R., Hosseinkhani, S., & Akhavan Sepahi, A., Isolation, identification and characterization of new luminous bacteria from Chah Bahar Port, southern marine habitat of Iran, *Iran J. Fish Sci.*, 14 (2015) 555-566.

9 Kannahi, M. & Sivasankari, S., Isolation and Identification of Bioluminescent Bacteria from Marine Water at Nagapattinam Sea Shore Area, *Int. J. Pharm. Sci. Rev. Res.*, 59 (2014) 346-351.

10 Wanjari, R. A., Shanware, A. S., Isolation and Characterization of Bioluminescent Bacteria, *IJAEMS*, (2017).

11 Kumar, A. R., Jayaprakashvel, M., FeuK-Lagerstedt, E., & Hussain, A. J., Factors Affecting Bioluminescence in Free Living *Photobacterium* spp. Isolated from Bay of Bengal, India, *J. Mar. Biosci*., 1 (2015) 33-49.

12 Malave-Orengo, J., Rubio-Marrero, E., & Rios-Velazquez, C., Isolation and characterization of bioluminescent bacteria from marine environments of Puerto Rico, *Technol. Edu. Top. Appl. Microbiol. Microbial Biotechnol.*, 1 (2010) 103-108.

13 Yaser, N. A., Abdullah, M. F. F., Aris, A. M., & Zainudin, I. I., Isolation and identification of bioluminescent bacteria in squid and water of Malaysia, *Int. J. Adv. Agric. Environ. Eng.*, 1 (2014) 225-228.

14 Amutha, K. & Shamini, S., Metal tolerance of bioluminescent bacteria isolated from marine organisms, *Indian J. Geo-Mar. Sci.*, 44 (2015) 1799-1806.

15 Arante, L. A. B., Sison, J. A. D., & Molina, A. J., A Report on Bioluminescent Bacteria Symbiont From Philippine Marine Fishes, *RJFH*, 10 (2015) 1-4.

16 Kulkarni, V. S. & Kulkarni, B. S., Isolation of Bioluminescent Bacteria and Their Application in Toxicity Testing of Chromium in Water, *Int. J. Curr. Microbiol. App. Sci*, 4 (2015) 23-32.

17 Makiguchi, N., Arita, M., & Asai, Y., Isolation, identification, and several characteristics of luminous bacteria, *JGAM*, 25 (1979) 387-396.

18 Campbell, A. K., Living light: biochemistry, function and biomedical applications, *Essays Biochem*., 24 (1989) 41-76.

19 Vishwakarma, A., Mathur, K., & Vuppu, S., Screening and tentative identification of bioluminescence producing bacteria in fish, *Int. J. Fish. Aquat. Stud.*, 1 (2014) (6) 225-231.

20 Dunlap, P., Bioluminescence microbiology, In: M. Schachter (Ed.), *Encyclopedia of Microbiology*, third edn., (Elsevier: Oxford, UK) 2009, 45-61.

21 Schwedt, G., Reiter, C., Uthemann, R., & Grabert, E., Development of an automated bacterial luminescence test for biomonitoring of environmental contaminants, *Fresenius J. Anal. Chem*., 359 (1997)155-160.

22 Applegate, B., Kelly, C., Lackey, L., McPherson, J., Kehrmeyer, S., Menn, F. M., Bienkowski, P., & Sayler, G., *Pseudomonas putida* B2: a tod-lux bioluminescent reporter for toluene and trichloroethylene co-metabolism, *J. Ind. Microbiol. Biot*., 18 (1997) 4-9.

23 Axelrod, T., Eltzov, E., & Marks, R. S., Bioluminescent bioreporter pad biosensor for monitoring water toxicity, *Talanta*, 149 (2016) 290-297.

24 Eltzov, E., Cohen, A., & Marks, R. S., Bioluminescent liquid light guide pad biosensor for indoor air toxicity monitoring, *Anal. Chem.*, 87 (2015) 3655-3661.

25 Shanware, A., Thakre, N., & Pande, S., Isolation and characterization of novel marine luminescent bacteria from Diu beach, India. *J. Pharm. Res.*, 7 (2013) 529-33.

26 Thakre, N., & Shanware, A., Screening of bioluminescent bacterial strains for developing novel toxicity sensing beads. *Conference Proceedings Paper–Sensors and Applications*; 2014.

27 Nawaz, A., & Ahmed, N., Isolation and characterization of indigenous luminescent marine bacteria from Karachi coast. *Acad. Res. Int*., 1 (2011) 74-83.

28 Thompson, F L., Iida, T., & Swings, J., Biodiversity of vibrios. *Microbiol Mol Biol R.*, 68 (2004) 403-31.

29 Delocado, E., Cruz, J., Barcelona, JR. E., Gamba, J., Aguyin, J., Siton, G., *et al*., Bioluminescent bacteria (*Vibrio fischeri* Pacini and *V. phosphoreum* Pacini) to direct a killing mechanism on leukemia cells.

30 Dunlap, P., Osmotic control of luminescence and growth in *Photobacterium leiognathi* from ponyfish light organs. *Arch Microbiol.*, 141 (1985) 44-50.

31 Carter, G. R., Isolation and identification of bacteria from clinical specimens, *Diagnostic procedure in veterinary bacteriology and mycology*, (1990) 19-39.

32 Garrity, G., Brenner, D., Krieg, N. & Staley, J., Bergey's Manual of Systematic Bacteriology volume 2, 2nd edn., *The Proteobacteria Part B: The Gammaproteobacteria*, (Springer: East Lansing) 2005, 491-546.

33 Hong, Y., Chen, Z., Zhang, B., & Zhai, Q., Isolation of Photobacterium sp. LuB-1 and its application in rapid assays for chemical toxicants in water. *Lett. Appl. Microbiol*., 51 (2010) 308-12.